Docket No.: 2912956-027000
Client Ref.: 349400 D21726 - LUV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.:	10/577,084
Applicant(s):	Gwenaëlle BESTEL-CORRE
Filed:	May 18, 2006
Art Unit:	1636
Examiner:	VOGEL, Nancy Treptow
For:	OPTIMISED MICRO-ORGANISM STRAINS FOR NADPH-CONSUMING BIOSYNTHETIC PATHWAYS

STATEMENT ACCOMPANYING TRANSLATION

EFS Filing
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed herein is an English Language Translation of French Application No. 0313056, filed November 6, 2003. Consistent with MPEP § 201.15, to Applicant's knowledge, "the translation of the certified copy is accurate."

Respectfully submitted,

Date: Date

Customer No.: **84331**

920 Massachusetts Avenue, N.W.
Suite 900
Washington, DC 20001

Telephone: 202-508-3400
Facsimile: 202-220-2213

The present invention concerns strains of microorganisms optimized for the production, by biotransformation, of substances with NADPH-consuming biosynthesis routes. The strains according to the invention can be used in biotransformation processes, and consume NADPH. The strains defined according to the invention can be prokaryotic or eukaryotic. In a preferred embodiment, the prokaryotic strain is a strain of *E. coli*. In a further embodiment the eukaryotic strain is a strain of *Saccharomyces*, in particular *S. cerevisiae*.

The present invention also concerns a process for the preparation of substances by biotransformation, through growth in an appropriate medium of a strain optimized according to the invention, which optimized strain also includes the genetic elements necessary for the preparation of such substances.

Biotransformation processes have been developed to allow the production of required substances in large quantities and at low cost, while at the same time making

profitable use of various industrial or agricultural by-products.

There are two main approaches for producing substances of interest by *in vivo* biotransformation:

1) Fermentation, whereby a substance is produced by a microorganism from a simple carbon source (*e.g.* WO0102547, which describes the production of lysine by fermentation of *C. glutamicum* in the presence of glucose).

2) Bioconversion by a microorganism of a given co-substrate into a molecule of interest (*e.g.* WO0012745, which describes the production of derivatives of R-piperidine, and WO0068397, which describes the production of tagatose). The co-substrate is not assimilated and is distinct from the carbon source, which is used solely to produce the biomass and the NADPH necessary for bioconversion.

The improvement of a biotransformation process can concern various factors such as temperature, oxygenation, medium composition, recovery process, etc. It can also be possible to modify a microorganism so as to increase the production and/or excretion of a molecule of interest.

In a fermentation approach, for example, the biosynthesis route can be improved, for example, by modifying gene regulation or by modifying genes to change the characteristics of the enzymes involved, or by optimizing the regeneration of cofactors.

In a bioconversion approach the emphasis will be placed on reducing the formation of by-products and

optimizing the regeneration of the cofactors involved in the bioconversion step or steps.

Among the cofactors involved in the biotransformations, NADPH is important in particular for the production of amino acids (*e.g.* arginine, proline, isoleucine, methionine, lysine), vitamins (*e.g.* pantothenate, phylloquinone, tocopherol), aromatics (*e.g.* WO9401564), polyols (*e.g.* xylitol), polyamines (*e.g.* spermidine), and other high added-value substances.

The present invention concerns a strain of microorganisms optimized for the production of substances with NADPH-consuming biosynthesis routes.

Instead of seeking to optimize the NADPH/$NADP^+$ ratio in the microorganism for each biotransformation, the inventors chose to produce modified microorganisms in order to obtain different NADPH/$NADP^+$ ratios, which modified microorganisms were then used to carry out NADPH-consuming biotransformations.

The principle of optimization of the NADPH/NADP ratio consists of limiting the enzyme activities involved in oxidation of NADPH notably to the benefit of reducing NAD as well as promoting enzyme activities for reducing NADP into NADPH. The activities involved in oxidation of NADPH to the benefit of reducing NAD by lowering, more particularly by deactivating the (UdhA, Qor) transhydrogenase activities. The enzyme activities for reducing NADP by setting the carbon flux via via the pentose phosphate cycle and/or by changing the co-factor specificity of an enzyme are promoted so that it uses NADP in preference to NAD, its usual cofactor.

According to the invention a strain of microorganism is taken to mean a set of microorganisms of the same species comprising at least one microorganism of that species. Thus the characteristics described for the strain apply to each of the microorganisms of that strain. Similarly, the characteristics described for any one of the microorganisms of the strain apply to the entire set of the microorganisms of that strain.

The microorganisms optimized according to the invention include bacteria, yeasts and filamentous moulds, and in particular bacteria and yeasts belonging to the following species: *Aspergillus sp.*, *Bacillus sp.*, *Brevibacterium sp.*, *Clostridium sp.*, *Corynebacterium sp.*, *Escherichia sp.*, *Gluconobacter sp.*, *Pseudomonas sp.*, *Rhodococcus sp.*, *Saccharomyces sp.*, *Streptomyces sp.*, *Xanthomonas sp.*, *Candida sp.*

The optimization of NADPH is described below for *E. coli* and *S. cerevisiae*. The same principle can be similarly applied to all microorganisms grown in aerobic conditions.

The optimized strains for producing NADPH (*i.e.* increased capability of reducing NADPH+) according to the invention comprise a deletion of the *udhA* gene and/or the *qor* gene. According to a preferential embodiment of the invention, the *udhA* and/or *qor* gene are both deleted.

By deletion is meant according to the invention, suppression of the "deleted" gene. This suppression may be an inactivation of the expression product of the relevant gene by suitable means, or inhibition of the expression of the relevant gene, or even of at least one

part of the relevant gene so that its expression is prevented (for example deleting part or all of the promoter region necessary for its expression), or so that the expression product loses its function (for example by deletion in the coding part of the gene concerned).

Preferentially, the deletion of a gene comprises the removal of that gene, and if required its replacement by a selection marker gene to facilitate the identification, isolation and purification of the strains optimized according to the invention.

According to a particular embodiment of the invention, the optimized strain according to the invention also comprises a deletion of a gene taken from *pgi* or *pfkA* and/or *pfkB* and/or a change of at least one gene taken from *lpd*, *gapA*: the change consisting of changing the preference of the enzyme to the benefit of NADP instead of NAD, its usual cofactor.

The strains according to the invention with deletion of the *pfkA* gene are more particularly suited for biotransformation processes.

To further increase the available amount of NADPH in the optimized microorganisms according to the invention, it may also be advantageous to overexpress at least one gene selected from *zwf*, *gnd*, *pntA*, *pntB*, *icd* and/or to delete at least one gene selected from *edd*, *aceA*, *aceB*, *aceK*.

The above genes are well known to one skilled in the art and described in the scientific literature, notably for *E. coli* and *S. cerevisiae*:

Genes and references in *E. coli*:

udhA: X66026 soluble pyridine transhydrogénase;

qor: L02312 quinone oxidoreductase;

pgi: X15196 phosphoglucose isomerase (EC 5.3.1.9);

*pfk*A: X02519 phosphofructokinase-1;

*pfk*B: K02519 phosphofructokinase-2;

edd: X63694 6-phosphogluconate dehydratase;

*ace*A: X12431 isocitrate lyase (EC 4.1.3.1);

*ace*B: X12431 malate lyase (EC 4.1.3.2);

*ace*K: M18874 isocitrate dehydrogenase kinase/phosphatase;

zwf: M55005 glucose 6-phosphate dehydrogenase;

gnd: K02072 6-phosphogluconate dehydrogenase;

*pnt*AB: X04195 pyridine nucleotide transhydrogenase subunits alpha and beta (EC 1.6.1.1);

icd: J02779 isocitrate dehydrogenase;

lpd: V01498 lipoamide dehydrogenase (=EC 1.8.1.4) involved in the pyruvate dehydrogenase complex;

*gap*A: AE000273 (201...1196) glyceraldehyde-3-phosphate dehydrogenase A (EC 1.2.1.12)

Genes and references in *S cerevisiae*:

*zta*1: NC_001134 NADPH:quinone reductase;

*pgi*1: NC_001134 phosphoglucoisomerase;

*pfk*1: M26943 phosphofructokinase, alpha subunit;

*pfk*2: M26944 phosphofructokinase, beta subunit;

*tdh*1: NC_001142 glyceraldehyde-3-phosphate-dehydrogenase;

*tdh*2: NC_001142 glyceraldehyde-3-phosphate-dehydrogenase;

*tdh*3: NC_001139 glyceraldehyde-3-phosphate-dehydrogenase.

The genes capable of being deleted or overexpressed for optimized strains according to the invention are mainly defined by using the naming of *E. coli*. However, this usage has a more general meaning according to the invention, and covers the corresponding genes of other microorganisms. Indeed, by using the GenBank references of genes of *E. coli*, one skilled in the art can identify the equivalent genes in bacterial strains other than *E. coli*.

The means of identifying homologous sequences and their percentages of homology are well known to those skilled in the art, and include, in particular, the BLAST program, which can be used from the website http://www.ncbi.nlm.nih.gov/BLAST/ with the default parameters indicated there. The sequences obtained can then be exploited (*e.g.* aligned) using, for example, the CLUSTALW program (http://www.ebi.ac.uk/clustalw/) or the MULTALIN program (http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl), with the default parameters indicated on their websites.

Alternatively, PFAMs or COGs may be used.

The PFAMs (Protein FAMilies database of alignments and Hidden Markov Models; http://www.sanger.ac.uk/Software/Pfam/) represent a large collection of alignments of protein sequences. Each PFAM makes it possible to visualize multiple alignments, see protein domains, evaluate distribution among organisms, access other databases, and visualize known protein structures.

The COGs (Clusters of Orthologous Groups of proteins; http://www.ncbi.nlm.nih.gov/COG/) are obtained by comparing protein sequences from 43 fully sequenced genomes representing 30 major phylogenetic lines. Each COG is defined from at least three lines, thus making it possible to identify ancient conserved domains.

It is possible to identify consensus sequences and design degenerate probes to clone the corresponding gene in another microorganism. These routine molecular biology methods are well known to those skilled in the art and are described, for example, in Sambrook *et al.* (1989 Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

Proteins analogous to soluble pyridine transhydrogenase coded by *udh*A of *E. coli*(P27306)

NP_709766 : soluble pyridine transhydrogenase [*Shigella flexneri* 2a str. 301]

E65203 : probable dehydrogenase (EC 1.8.1.-) *udh*A - *Escherichia coli* (strain K-12)

NP_463005: soluble pyridine nucleotide transhydrogenase [*Salmonella typhimurium* LT2]

Q8ZA97: soluble pyridine nucleotide transhydrogenase (STH) *Yersinia pestis*

P57112: soluble pyridine nucleotide transhydrogenase (STH) *Pseudomonas aeruginosa* PA01]

AF159108. soluble pyridine nucleotide transhydrogenase (sth) gene, *Azotobacter vinelandii*

AAN67764: soluble pyridine nucleotide transhydrogenase [*Pseudomonas putida* KT2440]

AAB50562: soluble pyridine nucleotide transhydrogenase [*Pseudomonas fluorescens*]

U91523: soluble pyridine nucleotide, *Pseudomonas fluorescens*

NC_003143: probable sthA, *Yersinia pestis* strain CO92,

P50529 : soluble pyridine nucleotide transhydrogenase *Vibrio cholera*

Proteins analogous to the quinone oxidoreductase coded by *qor* of *S.cerevisiae*(P38230, Zta1)

E877715: quinone oxidoreductase CC3759[imported] *Caulobacter crescentus*

T40981: probable quinone oxidoreductase - fission yeast (*Schizosaccharomyces pombe*) BAB48079: quinone oxidoreductase [*Mesorhizobium loti*]

A70871 : quinone oxidoreductase - *Mycobacterium tuberculosis* (strain H37RV)

NP_699755: quinone oxidoreductase [*Brucella suis* 1330]

BAC49848: quinone oxidoreductase [*Bradyrhizobium japonicum* USDA 110]

S52923: NADPH2: quinone reductase (EC 1.6.5.5) - *Pseudomonas aeruginosa*

AI0039: NADPH2: quinone reductase (EC 1.6.5.5) - *Yersinia pestis* (strain C092)

S45529: NADPH2, quinone reductase (EC 1.6.5.5) - *Escherichia coli* (strain K-12)

A further object of the present invention is a microorganism optimized for the production of NADPH as defined above and below, which microorganism also contains one or several coding genes of enzymes involved in the biotransformation of a molecule of interest, and one or several selection marker genes.

These genes can be native to the strain optimized by the invention, or be introduced into the strain optimized by the invention by conversion using a suitable vector, either by integration in the genome of the microorganism or by a replicative vector, which suitable vector bears one or several genes coding for the relevant enzymes involved in the biotransformation of the relevant molecule of interest and/or the relevant selection markers.

These genes include a nucleic acid sequence coding for an enzyme involved in the biotransformation of the

molecule of interest and/or for a selection marker, which coding sequence is merged with efficient promoter sequences in the prokaryote and/or eukaryote cell selected for biotransformation. The vector (or plasmid) can be a shuttle vector between *E. coli* and another microorganism.

The present invention also relates to a method for preparing optimized strains according to the invention as defined above and hereafter, wherein *udh*A and/or *qor* genes are deleted and if necessary a gene taken from *pgi* or *pfk*A and/or *pfk*B is deleted and/or at least one gene coding for NAD enzymes, in particular *lpd* and/or *gap*A, is modified so that they preferentially use NADP (Bocanegra, J.A. Scrutton, N.S.; Perham, R.N. (1993) Creation of an NADP-dependent pyruvate dehydrogenase multienzyme complex by protein engineering. *Biochemistry* **32**: 2737-2740), and if necessary at least one gene is deleted, selected from *edd*, *ace*A, *ace*B, *ace*K; these deletions and modifications being achieved by a suitable means, and/or at least one gene is overexpressed, selected from *zwf*, *gnd*, *pnt*A, *pnt*B, *icd* either by modifying the strain using a suitable vector that allows the overexpression, or by modifying the strength of the endogenous promoter controlling the gene that is to be overexpressed.

The selection of the strain optimized for the NADPH/NADP ratio will be determined according to the type of biotransformation (fermentation or bioconversion), to the total NADPH demand of the relevant bioconversion route, to the nature of the carbon source(s), to the biomass flux demand,…

The deletion of the gene *pgi* or *pfk*A and/or *pfk*B coding should be necessary if it is not possible to control the distribution of the carbon flux between glucolysis and the pentose phosphate pathway. The deletion of the *pgi* gene will be preferred for fermentations or when the demand for NADPH requires a minimum reduction flux of 2 moles of NADP per mole of imported glucose. The deletion of the *pfk*A gene will be chosen preferentially for bioconversions or when the demand for NADPH requires a minimum reduction flux of 3-4 moles of NADP per mole of imported glucose. The modification, as described above and below, of the *lpd* and/or *gap*A genes will be carried out to optimize the strains *E. coli* Δ(*udh*A, *qor*) or *E. coli* Δ(*udh*A, *qor*, *pgi*) or *E. coli* Δ(*udh*A, *qor*, *pfk*A, *pfkB*) and notably when biotransformations require a minimum reduction flow greater than 3 moles of NADP per mole of imported glucose. The other stated modifications, namely the deletion of at least one gene taken from *edd*, *ace*A, *ace*B, *ace*K or overexpression of at least one gene taken from *zwf*, *gnd*, *pnt*A, *pnt*B, *icd* may be carried out to fine-tune the optimization of the NADPH/NADP ratio to the needs of the cell and of the biotransformation process being considered.

According to a particular embodiment of the invention, the method for preparing strains according to the invention also comprises the transformation of optimized strains with at least one suitable vector comprising one or more genes coding for one or more enzymes involved in the biotransformation of a molecule of interest, as well as one or more selection marker genes.

The strain optimized according to the invention is obtained by molecular biology methods. One skilled in the art knows the protocols used to modify the genetic character of microorganisms. These methods are documented and are within the skills of one skilled in the art (Sambrook *et al.*, 1989 Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

The inactivation of a gene in *E. coli* is preferably carried out by homologous recombination (Datsenko K.A., Wanner B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**: 6640-6645). The principle of a protocol is briefly as follows: a linear fragment, obtained *in vitro*, comprising the two regions flanking the gene, and at least one selection gene located between these two regions (generally an antibiotic resistance gene), is introduced into the cell. This fragment thus contains an inactivated gene. Cells that have undergone a recombination event and integrated the introduced fragment are then selected by plating on a selective medium. Cells that have undergone a double recombination event, in which the wild gene has been replaced by the inactivated gene, are then selected. This protocol can be improved by using positive and negative selection systems, in order to speed up the detection of double recombination events.

The inactivation of a gene in *S. cerevisiae* is also performed preferentially by homologous recombination (Baudin *et al.*, *Nucl. Acids Res.* **21**, 3329-3330, 1993;

Wach *et al.*, *Yeast* **10**, 1793-1808, 1994; Brachmann *et al.*, *Yeast*. **14**:115-32, 1998).

The overexpression of a gene can be achieved by replacing the promoter of the gene *in situ* by a strong or inducible promoter. Alternatively, a replicative plasmid (single or multiple copy) is introduced into the cell in which the gene to be overexpressed is under the control of the appropriate promoter. In the case of modification of *Escherichia coli*, it is possible, for example, to use the promoters P*lac*-o, P*trc*-o, and p*tac*-o, three strong bacterial promoters for which the lac operator (*lac*O) has been deleted to make them constitutive. In the case of modifications of *Saccharomyces cerevisiae*, it is possible, for example, to use the promoters P*pgk*, P*adh*1, P*gal*1, P*gal*10.

A further aspect of the invention concerns the use of these strains optimized according to the invention for NADPH-dependent biotransformations, thereby obtaining an improved biotransformation yield compared with a strain not optimized for NADPH.

The biotransformations will be carried out using strains defined according to the invention in which genes will be expressed that code for enzymes catalyzing NADPH-dependent reactions. Those skilled in the art can easily identify such enzymes, as examples, let us mention the following enzymes without this list being limiting: EC 1.1.1.10 L-xylulose reductase, EC 1.1.1.51 3(or 17)□-hydroxysteroid dehydrogenase, EC 1.1.1.54 allyl-alcohol dehydrogenase, EC 1.1.1.80 isopropanol dehydrogenase, EC 1.1.1.134 dTDP-6-deoxy-L-talose 4-dehydrogenase, EC 1.1.1.149 20□-hydroxysteroid dehydrogenase, EC 1.1.1.151

21-hydroxysteroid dehydrogenase, EC 1.1.1.189 prostaglandin-E_2 9-reductase, EC 1.1.1.191 indole-3-acetaldehyde reductase EC 1.1.1.207 (-)-menthol dehydrogenase, EC 1.1.1.234 flavanone 4-reductase, EC 1.2.1.50 long-chain-fatty-acyl-CoA reductase, EC 1.3.1.4 cortisone □-reductase, EC 1.3.1.23 cholestenone 5□-reductase, EC 1.3.1.70 Δ^{14}-sterol reductase, EC 1.4.1.12 2,4-diaminopentanoate dehydrogenase, EC 1.5.1.10 saccharopine dehydrogenase, L-glutamate-forming, EC 1.7.1.6 azobenzene reductase, EC 1.8.1.5 2-oxopropyl-CoM reductase (carboxylating), EC 1.10.1.1 *trans*-acenaphthene-1,2-diol dehydrogenase, EC 1.14.12.3 benzene 1,2-dioxygenase, EC 1.14.12.8 4-sulfobenzoate 3,4-dioxygenase, EC 1.14.12.15 terephthalate 1,2-dioxygenase, EC 1.14.12.18 biphenyl 2,3-dioxygenase, EC 1.14.13.7 phenol 2-monooxygenase, EC 1.14.13.12 benzoate 4-monooxygenase, EC 1.14.13.26 phosphatidylcholine 12-monooxygenase, EC 1.14.13.64 4-hydroxybenzoate 1-hydroxylase, EC 1.14.13.70 sterol 14-demethylase, EC 1.16.1.5 aquacobalamine reductase, EC 1.17.1.1 CDP-4-dehydro-6-deoxyglucose reductase, EC 1.18.1.2 ferredoxin-NADP reductase.

The invention also concerns a process for producing a molecule of interest formed by a biosynthesis route that includes at least one NADPH-dependent reaction, characterized in that it comprises the following steps:

a) Growth in culture of microorganisms optimized according to the invention in an appropriate culture medium that favours their growth and that contains the substances necessary to carry out the

biotransformation by fermentation or bioconversion, except NADPH.

b) Extraction of the molecule of interest from the medium and its purification if necessary.

Preferably, the molecule of interest is selected from amino acids, vitamins, sterols, flavonoids, fatty acids, polyols, organic acids. Amino acids and their precursors include in particular lysine, methionine, threonine, proline, glutamic acid, homoserine, isoleucine, and valine. Vitamins and their precursors include in particular pantoate, trans-neurosporene, phylloquinone and tocopherols. Sterols include in particular squalene, cholesterol, testosterone, progesterone and cortisone. Flavonoids include in particular frambinone and vestitone. Organic acids include coumaric acid and 3-hydroxypropionic acid. Polyols include sorbitol, xylitol and glycerol.

In the case of a bioconversion, the process also includes the addition to the appropriate culture medium of the substrate that is to be converted.

The culture medium mentioned in step b) of the process according to the invention defined above contains at least one assimilable carbohydrate that can be any of various assimilable sugars, such as glucose, galactose, sucrose, lactose, molasses, or by-products of these sugars. A simple source of carbon that is especially preferred is glucose. Another preferred simple carbon source is sucrose. The culture medium can also contain one or more substances (*e.g.* amino acids, vitamins or mineral salts) that favour the growth of the

microorganism and/or the production of the molecule of interest. In particular, the mineral culture medium for *E. coli* can thus have a composition identical or similar to an M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* **32**:120-128), an M63 medium (Miller, 1992; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) or a medium such as that described by Schaefer *et al.* (1999, *Anal. Biochem.* **270**: 88-96).

The biotransformation conditions are set by those skilled in the art. In particular, the microorganisms are fermented at a temperature between 20°C and 55°C, preferably between 25°C and 40°C, and preferably at about 30°C for *S. cerevisiae* and at about 37°C for *E. coli*.

The following examples are given for illustration only and in no way restrict the embodiments or the scope of the invention.

EXAMPLES

Example 1: Optimizations to be carried out to improve the bioconversion of xylose into xylitol by *E.coli* and *S. cerevisiae*

a) Bioconversion with *E. coli*

Predictive modelling was carried out using the algorithm MetOpt®-Coli, a stoichiometric model developed by the company Metabolic Explorer, with which it was possible to determine 1) the maximum production yield of xylitol from xylose, and 2) the best flux distribution

from glucose to meet the needs for growth and redox equilibria that are necessary for the cell to grow and reach the maximum bioconversion yield.

Specific settings of model variables were 1) a glucose import flux of 3 $mmol.g^{-1}.h^{-1}$, 2) a variable growth rate of 0, 0.15 and 0.25 h^{-1}, 3) a variable membrane-bound transhydrogenase flux (*pnt*AB) less than or equal to 1 $mmol.g^{-1}.h^{-1}$; the limiting value of the membrane-bound transhydrogenase flux was determined from the literature (Hanson, 1979; Anderlund *et al.*, 1999; Emmerling *et al.*, 2002), and 4) a maintenance flux limited at between 5 and 22 $mmol.g^{-1}.h^{-1}$.

With the optimizations achieved according to the invention, the amount of NADPH which may be produced (by reduction of NADP), and so the xylitol bioconversion flux may be improved (Table 1). As further information, the theoretical maximum amounts of NADPH which may be produced (moles of NADPH produced per 3 moles of glucose) at $\mu=0$ are shown in the last column of Table 1. The theoretical maximum amounts of NADPH which may be produced for $\mu>0$ may be inferred by taking into account the needs and the productions related to the synthesis of biomass. When the biotransformation flux of xylose into xylitol is less than the amount of produced NADPH, this means that other factors (e.g. maintenance) limit biotransformation.

In all the cases produced, the model suggested the deletion of the *udh*A and *qor* genes. It is observed that the strain *E. coli* [Δ(*udh*A, *qor*)] allows optimum bioconversion of xylose into xylitol (given, not shown).

In practice, however, this strain does not afford a yield equivalent to the theoretical optimal yield, as it is difficult to maintain a suitable distribution of carbon flux between the pentose phosphate pathway and glycolysis, this distribution being variable according to the growth rate. In practice therefore, the *E. coli* strains No.8 [*gap*A-NADP-dependent, *lpd*A-NADP-dependent, Δ(*udh*A, *qor*, *pfk*A)] or No.4 [*gap*A-NADP-dependent, *lpd*A-NADP-dependent, Δ(*udh*A, *qor*, *pgi*)] are preferred, the choice between them depending on the growth rate of the strain during the bioconversion process. However, depending on the actual characteristics of the bioconversion process, other strains of the table may be considered, moreover it is possible to fine-tune the modelled strains, notably by making additional changes, such as overexpression of at least one gene taken from *zwf*, *gnd*, *pnt*A, *pnt*B and *icd* and/or deletion of at least one gene taken from *edd*, *ace*A, *ace*B, *ace*K.

No.	$\mu(h^{-1})$	0	0.15	0.25	*Amount of NADPH*
1	Δ*pgi*	5.47	5.21	3.67	12.00
2	Δ*pgi*+ *gap*A-NADP-dependent	12.87	10.91	7.28	12.00
3	Δ*pgi*+ *lpd*-NADP-dependent	17.00	10.39	1.99	17.00
4	Δ*pg*+ *gap*A-NADP-dependent *lpd*-NADP-dependent	20.58	14.88	9.99	22.00
5	Δ*pfk*A	20.27	13.94	0.56	28.00
6	Δ*pfk*A+ *gap*A-NADP-dependent	24.47	16.63	3.05	28.50
7	Δ*pfk*A+ *lpd*-NADP-dependent	25.00	16.79	5.32	31.00
8	Δ*pfk*A+ *gap*A-NADP-dependent *lpd*-NADP-dependent	28.00	19.13	7.80	32.50

Table 1: Theoretical optimal flux for converting xylose into xylitol (mmol.g^{-1}.h^{-1}) by strains of *E. coli* optimized for their NADPH reduction capacity

b) Bioconversion with *S. cerevisiae*

Predictive modelling was carried out using the algorithm MetOpt®-Scere, a stoichiometric model developed by the Company, with which it was possible to determine 1) the maximum production yield of xylitol from xylose, 2) the best flux distribution from glucose to meet the needs of the growth and redox equilibria necessary for the cell to grow and reach the maximum bioconversion yield.

Specific settings of model variables were 1) a glucose import flux of 3 mmol.g^{-1}.h^{-1}, 2) a variable growth rate of 0, 0.15 and 0.25 h^{-1}, 3) a maintenance flux de maintenance less than or equal to 22 mmol.g^{-1}.h^{-1}, 4) aldehyde dehydrogenase reactions (ALD2, ALD3, ALD6) irreversible and set in the direction acetate + NAD(P)H → acetaldehyde + NAD(P), and 5) no activities equivalent to *udh*A or *pnt*A,B.

The model allowed for mitochondrial and peroxisomal compartmentalization.

With the optimizations achieved according to the invention, the amount of NADPH which may be produced (by reduction of NADP) and so the xylitol bioconversion flux may be improved (Table 2). As further information, the theoretical maximum amounts of NADPH which may be produced (moles of NADPH produced per 3 moles of glucose)

at μ=0 are shown in the last column of Table 2. The theoretical maximum amounts of NADPH which may be produced for μ>0 may be inferred by taking into account the needs and the productions related to the synthesis of biomass. When the biotransformation flux of xylose into xylitol is less than the amount of produced NADPH, this means that other factors (e.g. maintenance) limit biotransformation.

In all the cases produced, the model suggested the deletion of the *zta*1 (ubiquinone oxidoreductase, homologous to *qor*). It is observed that one may use the optimized strain of *S. cerevisiae,* No.4 [*tdh*1,2,3-NADP-dependent, *lpd*1-NADP-dependent, Δ *zta*1)] wherein *tdh*1,2,3 corresponds to the *tdh*1, *tdh*2, *tdh*3 genes, respectively, which code for NAD-dependent glyceraldehyde 3-P dehydrogenase, the co-substrate specificity of which was changed to the benefit of NADP. However, in practice, with this strain, it should be difficult to provide the theoretical optimum yield, as it is difficult to maintain a suitable distribution of carbon flux between the pentose phosphate and glycolysis pathways, because this distribution varies with the growth rate. In practice, it is preferable to use the *E. coli* strain No.8 [*tdh*1,2,3-NADP-dependent, *lpd*1-NADP-dependent, Δ *zta*1, *pfk*A)]. Finally, it is possible to fine-tune the strains by carrying out changes, notably by making additional modifications, such as the overexpression of at least one gene selected from *zwf*, *gnd*, *pnt*A, *pnt*B and *icd* and/or the deletion of at least one gene selected from *edd*, *ace*A, *ace*B, *ace*K.

No.	$\mu(h^{-1})$	0	0.15	0.25	*Amount of NADPH*
1	Δ*pgi*	7.25	6.01	5.19	7.25
1	Δ*pgi*+ *tdh*1,2,3-NADP-dependent	12.67	10.51	9.08	12.67
3	Δ*pgi*+ *lpd1*-NADP-dependent	12.25	9.88	8.31	12.25
4	Δ*pg*+ *tdh*1,2,3-NADP-dependent *lpd*1-NADP-dependent	18.50	15.02	12.70	18.50
5	Δ*pfk*1,2	36.00	24.55	16.91	36.00
6	Δ*pfk*1,2+ *tdh*1,2,3-NADP-dependent	36.00	27.34	21.56	36.00
7	Δ*pfk*1,2+ *lpd*1-NADP-dependent	36.00	25.32	18.19	36.00
8	Δ*pfk*1,2+ *tdh*1,2,3-NADP-dependent *lpd*1-NADP-dependent	36.00	27.84	22.39	36.00

Table 2: Theoretical optimal flux for converting xylose into xylitol ($mmol.g^{-1}.h^{-1}$) by strains of *S.cerevisiae* optimized for their NADPH reduction capacity

Example 2: Construction of the strain *E. coli* [Δ(*udh*A, *qor*)]

The inactivation of the *udhA* and *qor* genes was achieved by inserting an antibiotic (chloramphenicol and kanamycin) resistance cassette while deleting most of the relevant gene. The method used is described by Datsenko, K.A.; Wanner B.L. in "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products", *Proc. Natl. Acad. Sci. USA*, **97**: 6640-6645).

For this purpose a pair of oligonucleotides was synthesized, each consisting of 100 pb of which 80 pb (lower case) are homologous with the gene to be deleted (*e.g. udhA*) and 20 pb are homologous with the cassette borne by the plasmids pKD3 and pKD4 and which are amplified by PCR using these oligonucleotides. The PCR product obtained was then introduced by electroporation into the *E. coli* strain [replicating the plasmid pKD46], which bears the gene coding for Red recombinase, which catalyzes homologous recombination. The resistant transformants were then selected and the insertion of the resistance cassette was checked by PCR analysis.

The chloramphenicol and kamamycin resistance cassettes may then be removed. To do this, the plasmid pCP20 carrying the FLP recombinase acting on the FRT

sites of the chloramphenicol or kamamycin resistance cassette was introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the antibiotic resistance cassette was checked by PCR analysis with the same oligonucleotides as those used earlier.

For practical reasons it can be useful to first delete one gene, suppress the antibiotic resistance gene and then delete the second gene.

The strain obtained was thus *E. coli* Δ(*udh*A, *qor*).

Example 3: Introduction of the p*xyl*1 plasmid coding for xylose reductase into the obtained strain and biotransformation of xylitol

The p*xyl*1 plasmid was constructed by insertion of the *xyl*1 gene into the Zero Blunt TOPO vector PCR cloning kit for sequencing (PCR4 TOPO vector, Invitrogen). To do this, the *xyl*1 gene (X59465) of *Pichia stipitis* was amplified by PCR with polymerase *Pwo* from chromosomal DNA.

The PCR product obtained was then directly cloned in the TOPO vector to obtain the p*xyl*1 plasmid. The TOPO vector carried a replication origin for *E. coli* and an ampicillin resistance gene and a kanamycin resistance gene.

The p*xyl*1 plasmid was then introduced into the strain *E. coli* DH5α for verification of the construction. The sequencing of the *xyl*1 gene of the p*xyl*1 plasmid with

the universal M13 forward and reverse oligonucleotides was then carried out to confirm the construction.

The validated plasmid was introduced into the strain *E. coli* Δ(*udh*A, *qor*) (Example 2) by electroporation.

The strain obtained, *E. coli* [Δ(*udh*A, *qor*) p*xyl*1], was then grown in fed batch, the initial medium (minimum medium) and the fed containing glucose and xylose. A strain of *E coli* [p*xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.
- The yield glucose/ xylitol.

It was found that the strain *E. coli* [Δ(*udh*A, *qor*), p*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

Example 4: Construction of the strain *E. coli* Δ(*udh*A, *qor*, *pgi*) and biotransformation

The inactivation of the *pgi* gene was carried out by using the method described in Example 2, except that the

initial strain is the strain of Example 2 instead of a wild strain.

The construction was carried out in rich medium (*e.g.* LB). The plasmid p*xyl*1 (Example 3) was then introduced by electroporation into the obtained *E. coli* Δ(*udh*A, *qor, pgi*) strain, and the resulting strain *E. coli* [Δ(*udh*A, *qor*, *pgi*) p*xyl*1] was selected on rich medium.

The strain obtained was then grown in fed batch, the initial medium (minimum medium) and the fed containing glucose and xylose. The strain of *E coli* [p*xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.
- The yield glucose/ xylitol.

It was found that the strain *E. coli* [Δ(*udh*A, *qor*), p*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

We may observe that the strain *E. coli* [Δ(*udh*A, *qor*), p*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

Example 5: Construction of the strain *E. coli* Δ(*udh*A, *qor*, *pfk*A) and biotransformation

The inactivation of the *pfk*A gene was carried out by using the method described in Example 2, except that the initial strain is the strain of Example 2 instead of a wild strain. The construction was carried out in rich medium (*e.g.* LB). The plasmid p*xyl*1 (Example 3) was then introduced by electroporation into the obtained strain *E. coli* Δ(*udh*A, *qor*, *pfk*A), and the resulting strain *E. coli* [Δ(*udh*A, *qor*, *pgi*, *pfk*A) p*xyl*1] was selected on rich medium.

The strain obtained *E. coli* [Δ(*udh*A, *qor*, *pgi*, *pfk*A) p*xyl*1] was then grown in fed batch, the initial medium (minimum medium) and the fed containing glucose and xylose. The strain *E coli* [p*xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.
- The yield glucose/ xylitol.

We may observe that the strain *E. coli* [Δ(*udh*A, *qor*, *pfk*A), p*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

Example 6: Construction of the strain *E. coli* [Δ(*udh*A, *qor*, *pfk*A, *lpd*) *lpd] and biotransformation**

The *lpd* gene coding for NADP-dependent dihydrolipoamide dehydrogenase, involved in the pyruvate dehydrogenase multienzyme complex, is deleted by using the method described in Example 2, except that the strain is strain described in Example 4 *E. coli* Δ(*udh*A, *qor*, *pgi*) instead of a wild strain. The construction and selection of the modified strain is carried out in a rich medium (*e.g.* LB). The obtained strain is *E. coli* Δ(*udh*A, *qor*, *pgi*, *lpd*).

Moreover, the plasmid p*lpd** is constructed which allows overexpression of a NADP-dependent dihydrolipoamide dehydrogenase. There are different possibilities for changing the the cosubstrate specificity of an enzyme. For instance, Bocanegra et al. (1993) disclose a method for producing a NADP-dependent dihydrolipoamide dehydrogenase.

The plasmids p*lpd** and p*xyl*1 were then introduced by electroporation into the strain *E. coli* [Δ(*udh*A, *qor*, *pgi*, *lpd*)], alternatively, it possible to choose cloning of *lpd** on p*xyl*1; the plasmid p*lpd***xyl*1 would then be obtained and introduced by electroporation into the strain *E. coli* Δ(*udh*A, *qor*, *lpd*). Construction and selection of the modified strain is carried out on rich medium (*e.g.* LB).

The obtained strain *E. coli* [Δ(*udh*A, *qor*, *pgi*, *lpd*) p*xyl*1, p*lpd**] was then grown in

in fed batch, the initial medium (minimum medium) and the fed containing glucose and xylose. The strain *E coli* [p*xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.
- The yield glucose/ xylitol.

We may observe that the strain *E. coli* [Δ(*udh*A, *qor*, *pgi*, *lpd*) p*xyl*1, p*lpd**] gave a greater production yield of xylitol than the non-optimized strain.

Example 7: Construction of the strain *S. cerevisiae* [Δ(*pgi*1) pRSGK-*xyl*1] and biotransformation

The pRSGK1-*xyl*1 plasmid results from the cloning of the *xyl*1 gene of *Pichia stiptis*, amplified by PCR by using a heat-resistant polymerase of *Pyrococcus woesi* (*Pwo*), in the pRSGK vector, under the control of the promoter P_{GK}. The plasmid is then introduced into *S. cerevisiae*. The obtained strain *S. cerevisiae* [pRSGK1-

*xyl*1] is then used for deleting the *pgi* gene. Inactivation of the *pgi* gene is carried out by inserting a marker (antibiotic resistance, auxotrophy) while deleting the major part of the relevant gene. The technique used is described by Brachmann C.B.; Davies, A.; Cost G.J.; Caputo, E.; Li, J.; Hieter, P.; Boeke, J.D. (1998) (Designer deletion strains derived from *Saccharomyces cerevisae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications, *Yeast*, **14**: 115-32); it is also possible to use the method described by Wach, A., Brachat, A., Pohlmann, R., et Philippsen, P. (1994), (New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*, *Yeast*, **10**: 1793-1808). In all the cases, a final strain *S. cerevisiae* [Δ*pgi* pRSGK1-*xyl*1].

Alternatively, it was also possible to choose to introduce the plasmid pRSGK-*xyl*1 into an available mutated strain, for example the Y23336 strain (EUROSCARF; http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/) heterozygous for the *pgi* gene (Mat α/a; *his*3D1/*his*3D1; *leu*2D0/*leu*2D0; *lys*2D0/*LYS*2; *MET*15/*met*15D0; *ura*3D0/*ura*3D0; *YBR196c*::*kan*MX4/*YBR196c*). It was then possible, after sporulation, to recover a homozygous strain *S. cerevisiae* [Δ(*pgi*) pRSGK- *xyl*1].

The obtained strain *S. cerevisiae* [Δ(*pgi*) pRSGK-*xyl*1] is then grown in fed-batch, the initial medium (supplemented YNB) and the fed containing glucose and xylose. Alternatively the initial medium may be a rich medium.

The control strain *S. cerevisiae* [Δ(*pgi*) pRSGK-*xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.
- The yield glucose/ xylitol.

We may observe that the strain *S. cerevisiae* [Δ(*pgi*) pRSGK-*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

Example 8: Construction of the strain *S. cerevisiae* Δ(*zta*1, *pgi*1) and biotransformation

The strain Y33183 (genotype: BY4743; Mat a/α; *his*3D1/*his*3D1; *leu*2D0/*leu*2D0; *lys*2D0/*LYS*2; *MET*15/*met*15D0; *ura*3D0/*ura*3D0; *YBR046c*::*kan*MX4/*YBR046c*::*kan*MX4) available from EUROSCARF is used and two *zta*1 genes (=YBR046c) of which are deleted. The strain is then transformed by the plasmid pRSGK-*xyl*1 (Example 7) and then deletion of the *pgi* gene is carried out according to the approach used in Example 7.

The obtained strain *S. cerevisiae* [Δ(*zta*1 *pgi*) pRSGK-*xyl*1] is then grown in fed-batch, the initial medium (YNB) supplemented with a glucose/xylose fed.

The control strain *S. cerevisiae* [pRSGK- *xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.
- The yield glucose/ xylitol.

We may observe that the strain *S. cerevisiae* [Δ(*zta*1 *pgi*) pRSGK-*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

Example 9: Construction of the strain *S. cerevisiae* [Δ(*zta*1, *pfk*1, *pfk*2) and biotransformation

The strain Y33183 (genotype: BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YBR046c::kanMX4/YBR046c::kanMX4) available from EUROSCARF is used and two *zta*1 genes (=YBR046c) of which are deleted. The strain is then transformed by the plasmid pRSGK-*xyl*1 (Example 7) and then deletions of the

*pfk*1 and *pfk*2 genes are carried out according to the approach used in Example 7.

One may possibly resort to the strain Y35893 [BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YGR240c::kanMX4/YGR240c::kanMX4] is used and the two *pfk*1 genes of which are deleted, and strain Y30791 [BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YMR20540c::kanMX4/YMR205c::kanMX4] and the two *pfk*2 genes of which are deleted. Both strains are available from EUROSCARF.

The obtained strain *S. cerevisiae* [Δ(*zta*1, *pfk*1, *pfk*2) pRSGK- *xyl*1] is then grown in fed-batch, the initial medium (supplemented YNB) and the fed containing glucose and xylose. Alternatively the initial medium may be a rich medium.

The control strain *S. cerevisiae* [pRSGK- *xyl*1] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase,
- The quantity of xylitol produced in the extracellular medium,
- The quantity of xylitol accumulated in the cells.
- The productivity in terms of xylitol.

- The yield glucose/ xylitol.

We may observe that the strain *S. cerevisiae* [D(*pgi*) pYES-*xyl*1] gave a greater production yield of xylitol than the non-optimized strain.

Example 10: Comparison between experimental values and predictions by metabolic model for optimizing production of xylitol by Escherichia Coli

Good correlation was observed between the predicyive modelling (Example 1) and the experiments described in Examples 3,4,5 and 6.

Examples 1-9 above are particular applications of the patent and do not limit the use thereof. One skilled in the art will easily be able to adapt these examples for the biotransformation of molecules having an NADPH-dependent synthesis. The MetOpt® algorithm and the strategy of optimizing an NADPH-dependent bioconversion process via optimization of the NADPH/NADP ratio is validated; we are then able to further claim a widened application to all NADPH-dependent biotransformations, which may be modelled and predicted by MetOpt® or one of its derivatives, with the use of *E. Coli* or *S. cerevisiae* or any other mictoorganism.

Example 11: Prediction of medications with which fermentation processes in E. Coli may be improved

Example 10 shows that the MetOpt® models developed by the Company are applicable to the bioconversions and

should be more generally applicable to biotransformations such as fermentations.

For example, MetOpt®-Coli model was applied to the production of cysteine (Table 3) or 3-hydroxypropionate (Table 4) by the fermentation of glucose by *E. coli*. We applied the same parameters as those used earlier, notably: 1) a glucose import flux of 3 $mmol.g^{-1}.h^{-1}$, 2) a variable growth rate of 0, 0.15 and 0.25 h^{-1}, 3) a variable membrane-bound transhydrogenase flux (*pnt*AB) less than or equal to 1 $mmol.g^{-1}.h^{-1}$; the limiting value of membrane-bound transhydrogenase flux was determined from the literature (Hanson, 1979; Anderlund *et al.*, 1999; Emmerling *et al.*, 2002), and 4) maintenance flux limited to between 5 and 22 $mmol.g^{-1}.h^{-1}$.

a) Case of production of cysteine by fermentation of glucose

In all the cases, the model suggests deletion of genes *udh*A and *qor*. It is observed that optimal production of cysteine may be achieved with the strain *E.Coli* No.8 [*gap*A-NADP-dependent, *lpd*-NADP-dependent, Δ(*udh*A, *qor*, *pgi*)]. Moreover, other strains may be just as interesting (e.g. strains Nos 2,3). Finally, it is possible to refine the modelled strains, notably by providing additional modifications, such as overexpression of at least one gene selected from *zwf*, *gnd*, *pntA*, *pntB*, and *icd* and/or deletion of at least one gene selected from *edd*, *aceA*, *aceB*, *aceK*.

No.	$\mu(h^{-1})$	0	0.15	0.25
1	Δ*pgi*	1.99	1.12	0.27
2	Δ*pgi*+ *gap*A-NADP-dependent	2.33	1.12	0.27
3	Δ*pgi*+ *lpd*-NADP-dependent	2.33	1.12	0.27
4	Δ*pg*+ *gap*A-NADP-dependent *lpd*-NADP-dependent	2.33	1.12	0.27
5	Δ*pfk*A	1.20	0.55	0.02
6	Δ*pfk*A+ *gap*A-NADP-dependent	1.85	0.89	0.19
7	Δ*pfk*A+ *lpd*-NADP-dependent	2.13	1.08	O.38
8	Δ*pfk*A+ *gap*A-NADP-dependent *lpd*-NADP-dependent	2.31	1.25	0.52

Table 3: Theoretical optimal flux for production of cysteine ($mmol.g^{-1}.h^{-1}$) by fermentation of glucose by *E.coli* strains optimized for their NADPH reduction capacity

b) Case of production of 3-hydroxypropionate by fermentation of glucose

In all the cases, the model suggests deletion of genes *udh*A and *qor*. It is observed that strain No.4 [*gap*A-NADP-dependent, *lpd*-NADP-dependent, Δ(*udh*A, *qor*, *pgi*)], finally other strains remain of interest (*e.g.* strains Nos. 2,3). Finally, it is possible to refine the modelled strains, notably by providing additional modifications, such as overexpression of at least one gene selected from *zwf*, *gnd*, *pntA*, *pntB*, and *icd* and/or deletion of at least one gene selected from *edd*, *aceA*, *aceB*, *aceK*

No.	$\mu(h^{-1})$	0	0.15	0.25
1	Δ*pgi*	4.00	2.37	0.89
2	Δ*pgi*+ *gap*A-NADP-dependent	5.29	2.98	0.89
3	Δ*pgi*+ *lpd*-NADP-dependent	5.45	2.98	0.89
4	Δ*pg*+ *gap*A-NADP-dependent *lpd*-NADP-dependent	5.47	2.98	0.89
5	Δ*pfk*A	4.86	1.99	0.08
6	Δ*pfk*A+ *gap*A-NADP-dependent	5.29	2.37	0.20
7	Δ*pfk*A+ *lpd*-NADP-dependent	5.38	2.52	0.20
8	Δ*pfk*A+ *gap*A-NADP-dependent+ *lpd*-NADP-dependent	5.38	2.52	0.20

Table 4: Theoretical optimal flux for production of 3-hydroxy propionate ($mmol.g^{-1}.h^{-1}$) by fermentation of glucose by *E.coli* strains optimized for their NADPH reduction capacity

Example 12: Predictions of the modifications for improving fermentation processes involving *S. cerevisiae*; application to the production of hydrocortisone

Example 10 shows that the MetOpt® models developed by the Company are applicable to bioconversions and should be more generally applicable to biotransformations such as fermentations.

For example, the MetOpt®-Scere model was applied to the production of hydrocortisone (Table 5) by fermentation of glucose by *S. cerevisiae*. We applied the same parameters as those used earlier, notably: 1) a glucose import flux of 3 $mmol.g^{-1}.h^{-1}$, 2) a variable growth rate of 0, 0.15 and 0.25 h^{-1}, 3) a maintenance flux less than or equal to 22 $mmol.g^{-1}.h^{-1}$, 4) reactions of aldehyde dehydrogenases (ALD2, ALD3, ALD6) irreversible and set in the direction acetate + NAD(P)H → acetaldehyde + NAD(P), and 5) the yeast has no activities equivalent to *udh*A or *pnt*A,B.

The model allows for mitochondrial and peroxisomal compartmentalization.

This representation of the results (Table 5) demonstrates the real contribution made by each of the mutations made according to the invention, to the improvement in NADPH production and so to the improvement in the hydrocortisone production flux.

No.	$\mu(h^{-1})$	0	0.15	0.25
1	Δ*pgi*	0.12	0.08	0.06
2	Δ*pgi*+ *tdh*1,2,3-NADP-dependent	0.21	0.14	0.10
3	Δ*pgi*+ *lpd1*-NADP-dependent	0.20	0.14	0.10
4	Δ*pg*+ *tdh*1,2,3-NADP-dependent *lpd*1-NADP-dependent	0.21	0.14	0.10
5	Δ*pfk*1,2	/	/	/
6	Δ*pfk*1,2+ *tdh*1,2,3-NADP-dependent	/	/	/
7	Δ*pfk*1,2+ *lpd*1-NADP-dependent	/	/	/
8	Δ*pfk*1,2+ *tdh*1,2,3-NADP-dependent + *lpd*1-NADP-dependent	/	/	/

Table 5: Theoretical optimal flux for the production of hydrocortisone (mmol.g^{-1}.h^{-1}) by fermentation of glucose by *S.cerevisiae* strains optimized for their NADPH reduction capacity)

In all the cases, the model suggests deletion of genes *udh*A and *qor*. It is observed that the *S. cerevisiae* strain No.2 [*gap*A-NADP-dependent, Δ(*udh*A, *qor*, *pgi*)], is of interest even if other strains may be just as interesting (*e.g.* strains Nos. 3,4). Moreover, it is always possible to refine the modelled strains, notably by providing additional modifications, such as overexpression of at least one gene selected from *zwf*, *gnd*, *pntA*, *pntB*, and *icd* and/or deletion of at least one gene selected from *edd*, *aceA*, *aceB*, *aceK*. Finally it is noted that strains from which the *pfk*1 and *pfk*2 genes are deleted are unable to produce hydrocortisone, and may not even be viable. This is because the production of hydrocortisone is limited more by carbon demand than by NADPH requirement. One solution is to allow a weak expression of a transhydrogenase type activity in the yeast. However, modelling shows that the hydrocortisone production will never be as high as when the *pgi*1 gene is deleted.

To further improve the theoretical optimum yield of the strains optimized according to the invention, additional modifications can be made, such as the overexpression of at least one gene that can be ZWF, SOL1, SOL2, SOL3, SOL4, GND1, GND2, IDP1, IDP2 or IDP3, and/or the deletion of at least one gene that can be either ICL1 or DAL7.

REFERENCES

Anderson, E.H. (1946) Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B", *Proc. Natl. Acad. Sci. USA* **32**:120-128

Baudin, A.; Ozier-Kalogeropoulos, O.; Denouel, A.; Lacroute, F. and Cullin, C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*, *Nucl. Acids Res.* **21**, 3329-3330

Bocanegra, J.A. Scrutton, N.S.; Perham, R.N. (1993) Creation of an NADP-dependent pyruvate dehydrogenase multienzyme complex by protein engineering. *Biochemistry* **32**: 2737-2740

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. **14:**115-32.

Datsenko, K.A.; Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**: 6640-6645

Miller, 1992; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sambrook et al. (1989 Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring

Harbor Lab., Cold Spring Harbor, New York

Schaefer U.; Boos, W.; Takors, R.; Weuster-Botz, D. (1999) Automated sampling device for monitoring intracellular metabolite dynamics, *Anal. Biochem.* **270**: 88-96

5 Wach, A.; Brachat, A.; Pohlmann, R.; and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*, Yeast 10, 1793-1808, 1994.

CLAIMS

1. A strain of a microorganisms characterized in that it comprises a deletion of the *udhA* gene and/or the *qor* gene and/or the *pgi* gene, with the proviso that when said microorganism is a bacterium, it comprises at least one deletion selected from the *udhA* gene and the *qor* gene.

2. The strain according to Claim 1 characterized in that it also comprises a deletion of at least one gene taken from a gene taken from *pgi* or *pfk*A and/or *pfk*B, and/or at least one gene is modified, coding for NAD enzymes, in particular *lpd* and/or *gap*A, so that they preferentially use NADP.

3. The strain according to either of Claims 1 and 2 characterized in that it has an overexpression of at least one gene selected from *zwf*, *gnd*, *pntA*, *pntB* and *icd*.

4. The strain according to Claims 1 to 3 characterized in that it comprises the deletion of at least one gene selected from *edd*, *aceA*, *aceB*, *aceK*.

5. The strain according to any of Claims 1 to 4 characterized in that it comprises one or more genes,

either endogenous or exogenous, coding for enzymes involved in the biotransformation of one molecule of interest.

6. The strain according to any of Claims 1 to 5 characterized in that it comprises one or more selection marker genes.

7. The strain according to any of Claims 1 to 6 characterized in that it is selected from bacteria, yeasts, and filamentary fungi.

8. The strain according to any of Claims 1 to 7, characterized in that it is selected from the following species: *Aspergillus sp.*, *Bacillus sp.*, *Brevibacterium sp.*, *Clostridium sp.* *Corynebacterium sp.*, *Escherichia sp.*, *Gluconobacter sp.*, *Pseudomonas sp.*, *Rhodococcus sp.*, *Saccharomyces sp.*, *Streptomyces sp.*, *Xanthomonas sp.*, and *Candida sp.*

9. A method for preparing optimized strains according to any of Claims 1 to 8, characterized in that the *udhA* and/or *qor* genes are deleted, and if necessary a gene taken from from *pgi* or *pfk*A and/or *pfk*B is deleted and/or and at least one gene coding for NAD enzymes, in particular *lpd* and/or *gap*A, is modified so that they preferentially use NADP, and if necessary at least one gene selected from *edd*, *aceA*, *aceB*, *aceK* is deleted;

these deletions and modifications being carried out by a suitable means and/or a gene selected from *zwf*, *gnd*, *pntA*, *pntB*, *icd* is overexpressed either by transforming the strain with a suitable vector comprising one or more genes coding for one or more enzymes involved in the biotransformation of a molecule of interest and/or one or more selection marker genes, or by modifying the force of an endogenous promoter controlling the gene to be expressed.

10. A method for producing a molecule of interest of which at least one of the reactions of the biosynthesis routes is NADPH-dependent characterized in that it comprises the following steps:

c) growth in a culture of micro-organisms optimized according to any of Claims 5 to 8 in an appropriate culture medium that favours their growth and contains the required substances for carrying out biotransformation by fermentation or bioconversion, with the exception of NADPH, and

d) extraction of the molecule of interest from the medium and if necessary its purification.

11. A method according to Claim 10 characterized in that the molecule of interest is selected from amino acids, vitamins, sterols, flavonoids, fatty acids, polyols and organic acids.